Second Serogroup of *Legionella feeleii* Strains Isolated from Humans

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Three strains of *Legionella feeleii* from patients with pneumonia (425-MI-H, 691-WI-H, and 693-WI-H) and one environmental strain (713-MI-E) received at the Centers for Disease Control for reference diagnostic testing were compared with the type strain WO-44C-C3 (ATCC 35072) by DNA hybridization, chemical analysis of cellular fatty acids and ubiquinones, biochemical tests, and serological characteristics. All four isolates were assigned to the *L. feeleii* species on the basis of DNA hybridization results. However, strains 691-WI-H and 693-WI-H were serologically distinct from strain WO-44C-C3, as shown by their minimal reactivity (1 to 2+) with a direct immunofluorescence conjugate prepared against *L. feeleii* serogroup 1 (strain WO-44C-C3). Therefore, strains 691-WI-H and 693-WI-H were placed in a new *L. feeleii* serogroup (serogroup 2). The reference strain of *L. feeleii* serogroup 2 is 691-WI-H (ATCC 35849).

There are currently 22 *Legionella* species comprising 32 serogroups (2). Three species *L. pneumophila*, *L. longbeachae*, and *L. bozemanii* contain two or more serogroups each. *Legionella* have been isolated or specimens have been positive by the direct immunofluorescence assay (DFA) from patients infected with eight of the species (*L. pneumophila*, *L. bozemanii*, *L. dumoffii*, *L. micdadei*, *L. longbeachae*, *L. jordanis*, *L. waterworthii*, and *L. hackeliae*). So far, strains of the other 14 species have been reported only from environmental specimens. *L. feeleii* WO-44C-C3 is an example of the latter situation. Seroconversions in patients with Pontiac fever were documented against an indirect immunofluorescence assay (IFA) antigen of strain WO-44C-C3 (6), but the bacterium was not isolated from specimens from patients. When a DFA conjugate to strain WO-44C-C3 became available, we began testing specimens from patients with suspected legionellosis for possible *L. feeleii* infection. In this report, we describe four new *L. feeleii* strains, three of which were isolated from patients with pneumonia. Two of the isolates were serologically distinct from the type strain and, therefore, were placed in a new *L. feeleii* serogroup (serogroup 2).

**MATERIALS AND METHODS**

*Strains.* The *L. feeleii* strains, source, and location of isolation are given in Table 1. The three strains isolated from human specimens were from three different patients with pneumonia and were submitted to the Centers for Disease Control through the respective state health laboratories. The type strain of *L. feeleii* (WO-44C-C3 [ATCC 35072]) was isolated from water during an outbreak investigation and has been described previously (6).

DFA. The four new *L. feeleii* isolates were tested with working dilutions of the following DFA conjugates: *L. pneumophila* serogroups 1 through 9, *L. longbeachae* serogroups 1 and 2, *L. dumoffii*, *L. micdadei*, *L. gormanii*, *L. bozemanii* serogroup 1, *L. jordanis*, *L. waterworthii*, *L. oakridgensis*, *L. sainthelensi*, and *L. feeleii* (WO-44C-C3).

**Biochemical characteristics.** The *L. feeleii* strains were tested for the absence of growth on Trypticase soy agar containing 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.) and buffered charcoal yeast extract (BCYE) agar (13) without L-cysteine. The hydrolysis of sodium hippurate was performed by the method of Hébert (5). The strains were grown on charcoal-yeast extract agar plates (4) and tested for autofluorescence with a Woods lamp set at a wavelength of 366 nm. The chromogenic cephalosporin test for the detection of beta-lactamase production was done as described by Thornberry and Kirven (16). Ryu stain was used to demonstrate flagella (7). Urea hydrolysis was determined by inoculating Christensen urea-agar slants. Gelatin hydrolysis was determined with BCYE medium in which the agar was replaced with 3% gelatin. Methods described by Weaver and Feeley were used for oxidase, catalase, nitrate reduction, and fermentation tests (17). Browning of yeast extract agar medium supplemented with 1% L-tyrosine was determined as described by Orrison et al. (14). The cellular fatty acid composition and ubiquinone content of each isolate was determined by methods described previously (9, 10, 12).

**DNA hybridization.** Strains were grown until growth was confluent (3 to 5 days), at 35°C in an atmosphere of 2.5% CO2 on 15 to 30 petri plates (100 by 15 mm) that contained BCYE agar. The preparation of unlabeled DNA has been described, as has the determination of DNA relatedness by the hydroxyapatite method at 60 and 75°C (1). DNA from strains 425-MI-H, 691-WI-H, and 693-WI-H were labeled in vitro with 32P-labeled deoxyctydosine triphosphate provided in a Nick Translation Kit (catalog no. 8160 SB; Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Labeling was done according to the directions of the manufacturer. Labeled DNA was purified by passage over a Bio-Gel P-100 column (Bio-Rad Laboratories, Richmond, Calif.) equilibrated in 0.015 M NaCl-0.0015 M sodium citrate.

**Preparation of antisera.** Antisera were prepared for the WO-44C-C3, 691-WI-H, and 693-WI-H strains by methods described previously (15). For the slide agglutination test (SAT), antiserum titers were determined by preparing two-
425-MI-H
Sputum
Lung tissue
693-WI-H
691-WI-H
Legionella cells
phosphate-buffered saline
The temperature cells a
30
suspended in 2.0 ml of 10% (vol/vol) neutral
75 mm)
and heated in
WI-H,
SAT,
0.5%.
Twofold
reacted
4+
stained strains
physiological characteristics were
negative
with
(1
to
classified
as
serogroup
1,

Biochemical characteristics. All four new L. feeleii strains
and the type strain (WO-44C-C3) were gram-negative rods
which did not grow on Trypticase soy blood agar or on
BCYE agar without L-cysteine. All strains were negative in
tests for reduction of nitrates, urease, carbohydrate
fermentation, gelatin liquefaction, beta-lactamase, and
auto-fluorescence. They were positive in tests for catalase, and
each possessed flagella. In tests for oxidase and browning of
yeast extract agar with tyrosine, the reactions varied from
negative to weakly positive.

Chemical composition. The nonhydroxylated and
monohydroxylated fatty acid profiles of all strains were
consistent with those published previously for L. feeleii (8,
10, 11). No dihydroxylated fatty acids were found in any
of these strains. Ubiquinone-13 and ubiquinone-14 were the
only quinones detected in more than trace amounts; the
relative amount of ubiquinone-13 was approximately twice
that of ubiquinone-14 in each isolate.

DNA relatedness. DNA relatedness values of the four new
strains to each other, to the type strain of L. feeleii, and to
other Legionella species are shown in Table 2. Relatedness
to each other and to the L. feeleii type strain was 71 to 98%
in 60°C reactions, and when allowed to react at 75°C related-
ness was 78 to 100%. The divergence (percent unpaired
bases) in related sequences was 0.0 to 0.5%. Relatedness of
these strains to type strains of other Legionella species
was 1 to 15%.

TABLE 1. L. feeleii strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>WO-44C-C3</td>
<td>Environmental</td>
<td>Ontario, Canada</td>
</tr>
<tr>
<td>425-MI-H</td>
<td>Sputum</td>
<td>Michigan</td>
</tr>
<tr>
<td>713-MI-E</td>
<td>Environmental</td>
<td>Michigan</td>
</tr>
<tr>
<td>691-WI-H</td>
<td>Lung tissue</td>
<td>Wisconsin</td>
</tr>
<tr>
<td>693-WI-H</td>
<td>Lung tissue</td>
<td>Wisconsin</td>
</tr>
</tbody>
</table>

RESULTS

DFA results. A conjugate prepared against L. feeleii
serogroup 1, strain WO-44C-C3, and used at its working
dilution stained strains 691-WI-H and 693-WI-H cells weakly
(1 to 2+). Strains 425-MI-H and 713-MI-E were each stained
4+ with the conjugate and were therefore serologically
classified as serogroup 1 isolates. All four strains were
negative with all other available DFA conjugates. Studies
therefore were undertaken to determine the genetic and
physiological characteristics of these strains and to
determine whether strains 691-WI-H and 693-WI-H represented
a new L. feeleii serogroup.

Biochemical characteristics. All four new L. feeleii strains
and the type strain (WO-44C-C3) were gram-negative rods
which did not grow on Trypticase soy blood agar or on
BCYE agar without L-cysteine. All strains were negative in
tests for reduction of nitrates, urease, carbohydrate
fermentation, gelatin liquefaction, beta-lactamase, and
auto-fluorescence. They were positive in tests for catalase, and
each possessed flagella. In tests for oxidase and browning of
yeast extract agar with tyrosine, the reactions varied from
negative to weakly positive.

Chemical composition. The nonhydroxylated and

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SAT. The *L. feeleii* strains were tested with the SAT to determine their within-species antigenic relationship and to reveal cross-reactions with other *Legionella* species. Antisera were prepared against *L. feeleii* WO-44C-C3, 691-WI-H, and 693-WI-H and against all previously characterized legionellae: the 20 strains listed above and *L. spiritensis*, *L. hackeliae*, *L. maceachernii*, *L. jamestowniensis*, *L. sainticruis*, *L. cherrii*, *L. steigerwaltii*, *L. parisiensis*, *L. rubrilucens*, *L. erythra*, *L. anisa*, and *L. bozemanii* serogroup 2. No cross-reactions of *L. feeleii* isolates were observed other than with the *L. feeleii* antisera.

Reciprocal slide agglutinating antibody titers of unabsorbed and absorbed rabbit antisera against strains WO-44C-C3 and 691-WI-H are shown in Table 3. Absorption of each antisera with the heterologous serogroup strain removed agglutinating antibodies against the absorption strain but not antibodies against the homologous serogroup strain. Similar results were obtained with the 693-WI-H antisera and 693-WI-H strain (not shown). In addition, repeated absorptions of 691-WI-H antisera with 693-WI-H cells (two times), and 693-WI-H strain with cells (three times), removed all *L. feeleii* agglutinating antibodies present in the sera, indicating that the two isolates have homologous agglutinating antigens. Strains 425-MI-H and 713-MI-E reacted 4+ with absorbed WO-44C-C3 antisera but not with absorbed 691-WI or 693-WI-H antisera, indicating that these three isolates are homologous serologically.

IFA. Brown et al. (3) demonstrated several advantages of using an IFA instead of DFA to identify *Legionella* strains, including conservation of reagents and greater sensitivity. Table 4 shows the IFA antibody titers of the unabsorbed and absorbed *L. feeleii* antisera. As shown previously with SAT, homologous titers were unaffected by absorption with cells of the heterologous serogroup, whereas the heterologous titers were greatly reduced after absorption.

TABLE 3. Slide agglutinating antibody titers of unabsorbed and absorbed rabbit antisera against *L. feeleii* strains

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Absorbed with strain of</th>
<th>SAT titer* against antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Serogroup 1</td>
</tr>
<tr>
<td>Serogroup 1</td>
<td></td>
<td>64</td>
</tr>
<tr>
<td>Serogroup 2</td>
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<td>64</td>
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<tr>
<td>Serogroup 2</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Serogroup 1</td>
<td></td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

* Dilution giving 2+ or greater agglutination. Serogroup 1, Strain WO-44C-C3; serogroup 2, strain 691-WI-H.

TABLE 4. IFA antibody titers of unabsorbed and absorbed rabbit antisera against *L. feeleii* strains

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Absorbed with strain of</th>
<th>IFA titre* against antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Serogroup 1</td>
</tr>
<tr>
<td>Serogroup 1</td>
<td></td>
<td>16,384</td>
</tr>
<tr>
<td>Serogroup 2</td>
<td></td>
<td>16,384</td>
</tr>
<tr>
<td>Serogroup 2</td>
<td></td>
<td>2,048</td>
</tr>
<tr>
<td>Serogroup 1</td>
<td></td>
<td>&lt;8</td>
</tr>
</tbody>
</table>

* Highest dilution giving at least 1+ fluorescence intensity. Serogroup 1, Strain WO-44C-C3; serogroup 2, strain 691-WI-H.

Isolates from both serogroups of *L. feeleii* were obtained from patients with pneumonia.

It is noteworthy that all strains of *L. feeleii* serogroup 1 identified so far were from Michigan or an adjacent area (Windsor, Ontario, Canada). Included are the one human and two environmental isolates described in this study and another human isolate received from Michigan since completion of the experiments described herein. In addition, both of the *L. feeleii* serogroup 2 isolates, also from specimens from patients, were from Wisconsin, a neighboring state. Whether this close geographical clustering of the first six isolates of *L. feeleii* represents an endemic distribution of the species remains to be determined.

In a previous report (15), complete agreement of results in testing *Legionella* isolates with DFA and SAT was shown. Several advantages of the SAT include simpler and less expensive reagent preparation, ease of performance, and the fact that no instruments are required to obtain the results. These factors are even more relevant now, as the number of *Legionella* species has recently tripled. In another, more comprehensive study of the antigenic relationships of the 22 *Legionella* species (14a), we show that pooled antisera can be used in a test for the 33 serogroups currently recognized.

ACKNOWLEDGMENTS

We thank William Bibb for providing the *L. oakridgensis*, *L. sainthelenis*, and *L. feeleii* (WO-44C-C3) conjugates used in this study. We also thank Joan Nagel for secretarial assistance.

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LITERATURE CITED